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Cap-specific mRNA (nucleoside-$O^{2'}$-)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein

(mRNA processing/methylation/polyadenylylation/poxvirus)

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ABSTRACT **The vaccinia virus gene for *S*-adenosyl-L-methionine:mRNA (nucleoside-$O^{2'}$-)-methyltransferase, an enzyme required for the formation of the 5′ cap structure of mRNA, was identified. Protein sequence analysis revealed that this cap-specific methyltransferase is derived from the same open reading frame as that previously shown to encode VP39, a M_r 39,000 dissociable subunit of poly(A) polymerase that stimulates the formation of long poly(A) tails. Consistent with this finding, methyltransferase activity was associated with the heterodimeric poly(A) polymerase, which is composed of VP55 and VP39 subunits, as well as with monomeric VP39 protein isolated from vaccinia virions. In addition, cap-specific nucleoside-$O^{2'}$-methyltransferase activity is associated with recombinant VP39, which was purified to near homogeneity from mammalian cells. From these data, we concluded that the same protein functions as a methyltransferase and a poly(A) polymerase stimulatory factor to modify the 5′ and 3′ ends of mRNA, respectively.**

mRNA precursors of eukaryotes and their viruses are processed extensively during and after transcription. These modifications include the addition of a 5′ terminal cap structure and a 3′ terminal poly(A) tail. The cap, which is added cotranscriptionally, stabilizes the mRNA and facilitates the binding of translation initiation factors and ribosomes. Three activities modify the RNA 5′ terminus to form the $m^7G(5')pppN^m$ cap structure: mRNA guanylyltransferase (1–3), mRNA (guanine-N^7-)-methyltransferase (1, 4), and mRNA (nucleoside-$O^{2'}$-)-methyltransferase (EC 2.1.1.57) (5, 6). Capping begins with the transfer of a GMP residue from GTP to the 5′ diphosphate end of the nascent mRNA to form a unique 5′-5′ phosphodiester linkage. A methyl group is then transferred from *S*-adenosylmethionine (AdoMet) to the N^7 position of the added guanine moiety to create a $m^7G(5')pppG$ structure (cap 0). Next, a methyl group from AdoMet is transferred to the 2′ position of the ribose of the penultimate nucleoside, to form $m^7G(5')pppN^m$ (cap I). The latter methylation is cap 0 specific, consistent with ordered addition of methyl groups during the formation of the cap I structure (7). The cap I structure can be further modified by a cap-specific mRNA ($O^{2'}$-methyladenosine-N^6-)-methyltransferase (8) and by a second mRNA (nucleoside-$O^{2'}$-)-methyltransferase to form $m^7G(5')pppN^m$-N^m (cap II; ref. 6). Cap I and II structures form the 5′ ends of the mature mRNAs of most higher eukaryotes and their viruses, whereas cap 0 is present in lower eukaryotes and plants (9). Although the importance of the terminal m^7G in the stability and translation of mRNA has been well documented, the roles of the additional cap methylations of eukaryotic mRNA remain unknown.

Viruses have provided important model systems for the study of mRNA synthesis and processing. Poxviruses, of which vaccinia virus is the prototype, are large DNA viruses that encode a complete expression system, including a eukaryotic-like multisubunit DNA-dependent RNA polymerase, transcription factors, and mRNA-modifying enzymes that are necessary for viral replication in the cytoplasm of the cell (10). The proteins required for expression of the early regulatory class of genes are packaged in the infectious virus particle, whereas those for the intermediate and late regulatory classes of genes are synthesized in a cascade fashion just before their use. The genes for many of these proteins, including RNA polymerase subunits (11–18), transcription factors (19–22), mRNA guanylyltransferase/guanine-N^7-methyltransferase (23, 24) and poly(A) polymerase (25) have been identified. For some enzymes, such as the mRNA guanylyltransferase/guanine-N^7-methyltransferase, the corresponding cellular genes have not yet been described. The gene encoding mRNA (nucleoside-$O^{2'}$-)-methyltransferase was not known for either poxviruses or eukaryotes, prompting the present study.

We now report the identification of the mRNA (nucleoside-$O^{2'}$-)-methyltransferase gene of vaccinia virus. Surprisingly, the same gene was previously found to encode VP39, the stimulatory subunit of poly(A) polymerase (25). Consistent with this finding, the purified vaccinia virus poly(A) polymerase heterodimer as well as recombinant VP39 monomer have cap-specific mRNA methyltransferase activity.

MATERIALS AND METHODS

Methyltransferase Assay. The 0.1-ml reaction mixture contained 25 mM Hepes/NaOH (pH 7.5), 1 mM dithiothreitol, 1 μM Ado[*methyl*-^{3}H]Met (6.9 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and 5 μg of RNA isolated from brome mosaic virus (BMV; supplied by A. Hadidi, U.S. Department of Agriculture, Beltsville, MD). After incubation with the enzyme for 30 min at 37°C, the reactions were stopped by boiling the samples for 2 min. Incorporation of [^{3}H]methyl groups into RNA was determined by a filter-binding assay (5).

Methyltransferase Purification. The enzyme was purified essentially as described (5). Briefly, core particles from 40 mg of purified vaccinia virus (strain WR) were disrupted with deoxycholate, NaCl, and dithiothreitol and passed through a DEAE-cellulose column primarily to remove nucleic acids. The column effluent was dialyzed against 50 mM sodium acetate (pH 6.0), 0.2 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol and then loaded on a CM-Sepharose column that had been equilibrated with the same buffer. Protein was eluted with a linear gradient of 0.2–0.6 M NaCl, and fractions were assayed for methyltransferase activity. The peak fractions were pooled, heated with

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Abbreviations: AdoMet, *S*-adenosylmethionine; BMV, brome mosaic virus; ORF, open reading frame.

mercaptoethanol and SDS, and analyzed by PAGE. The electrophoretically separated proteins were transferred to a nitrocellulose membrane and the 38-kDa band was detected by staining with ponceau S dye and excised.

Sequence Analysis. Sequencing was done at the Harvard Microchemistry Laboratory under the direction of William Lane. The 38-kDa protein was digested *in situ* with trypsin, and the peptides were isolated by reverse-phase HPLC on a C_{18} column. N-terminal sequence analysis was done with an Applied Biosystems 477A sequencer with a 120A on-line phenylthiohydantoin-amino acid analyzer. Protein data base searches were conducted with the programs of Pearson and Lipman (26).

Purification of Recombinant VP39. Open reading frame (ORF) J3R was overexpressed using the hybrid vaccinia virus/bacteriophage T7 RNA polymerase system (27, 28). VP39 was purified from 5×10^9 HeLa cells in five chromatographic steps ending with poly(A) Sepharose (detailed by P.D.G. and B.M., unpublished work).

Immunoblot Analysis. Immunoblots were done as described (25).

Analysis of Methylated Cap Dinucleotides and Nucleosides. BMV RNA (10 μg) was incubated in a standard methyltransferase assay and separated from Ado[*methyl*-^{3}H]Met by passage through a Sephadex G50 column in 0.05 M ammonium acetate. The purified RNA was lyophilized and digested with 23 units of nuclease P1 (BRL) in 50 μl of 50 mM sodium acetate for 2 hr at 37°C and then with 20 units of calf intestinal alkaline phosphatase (BRL) in 50 μl of 50 mM Tris·HCl, pH 8.6/5 mM $MgCl_2$ for 2 hr at 37°C. The digest was applied directly to a cellulose thin-layer sheet (Eastman). Ascending chromatography was done in isobutyric acid/0.5 M NH_4OH, 5:3. After the sheet was dried, the positions of authentic standards were determined by examination under UV light. The [^{3}H]methyl-labeled substances were detected by counting 1-cm strips in a toluene-based scintillation fluid.

To identify the methylated nucleosides, the labeled BMV RNA was digested with 5 units of snake venom phosphodiesterase (Pharmacia) and 20 units of calf intestinal alkaline phosphatase in 50 μl of 50 mM Tris·HCl, pH 8.5/5 mM $MgCl_2$ for 2 hr at 37°C. Thin-layer chromatography was done as described above, except that ethyl acetate/2-propanol/7.5 M NH_4OH/1-butanol, 3:2:2:1, was used as a developing solvent.

RESULTS

Identification of the mRNA (nucleoside-$O^{2'}$-)-methyltransferase Gene. A specific assay for vaccinia virus mRNA (nucleoside-$O^{2'}$-)-methyltransferase was devised (5) using Ado[*methyl*-^{3}H]Met and BMV genomic RNA, which consists of four single-stranded RNA molecules each beginning with the sequence m^{7}G(5′)pppG (29), as the methyl donor and acceptor, respectively. The product of this reaction was shown to be exclusively m^{7}G(5′)pppG^m. Enzymes were extracted from vaccinia virus core particles and purified by DEAE-cellulose and CM-Sepharose column chromatography steps. As described earlier (5), the methyltransferase activity cochromatographed with a 38-kDa protein (data not shown). The peak activity fractions from the CM-Sepharose column were pooled, and the 38-kDa protein was isolated by SDS/PAGE and transferred to a nitrocellulose membrane. After *in situ* trypsin digestion and separation of the peptides by HPLC, three peptides were sequenced. Comparisons with a library of vaccinia virus protein sequences revealed that all three peptides perfectly matched segments of a single ORF previously designated J3R (Fig. 1). Surprisingly, this ORF was previously shown to encode VP39, the stimulatory subunit of vaccinia virus poly(A) polymerase (25).

Poly(A) Polymerase Heterodimer and Its Simulatory Subunit Have Methyltransferase Activity. The poly(A) polymerase comprises a heterodimer of two polypeptides with molecular masses of ≈55 and 39 kDa (30). VP55 catalyzes primer-dependent poly(A) synthesis, whereas the smaller subunit, VP39, stimulates the formation of long poly(A) tails (25). Column fractions from a single-stranded DNA-cellulose column, obtained during the final stages of purification of poly(A) polymerase from virions (25), were used to assay methyltransferase activity. The methyltransferase eluted in two distinct peaks (Fig. 2). The first peak corresponded with the VP39 monomer detected by immunoblotting. The second peak of methyltransferase coeluted with active poly(A) poly-

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MDVVSLDKPFMYFEEIDNELDYEPESANEVAKKLPYQGQLKLLLGELFFL  50
SKLQRHGILDGATVVYIGSAPGTHIRYLRDHFYNLGVIIKWMLIDGRHHD 100
PILNGLRDVTLVTRFVDEEYLRSIKKQLHPSKIILISDVRSKRGGNEPST 150
ADLLSNYALQNVMISILNPVASSLKWRCPFPDQWIKDFYIPHGNKMLQPF 200
APSYSAEMRLLSIYTGENMRLTRVTKSDAVNYEKKMYYLNKIVRNKVVVN 250
FDYPNQEYDYFHMYFMLRTVYCNKTFPTTKAKVLFLQQSIFRFLNIPTTS 300
TEKVSHEPIQRKISSKNSMSKNRNSKRSVRSNK  333
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FIG. 1. Peptide sequences of the mRNA (nucleoside-$O^{2'}$-)-methyltransferase match segments of the J3R ORF. Tryptic peptides derived from the purified 38-kDa mRNA (nucleoside-$O^{2'}$-)-methyltransferase were isolated by HPLC, and material from three well isolated peaks were sequenced. The peptide sequences obtained are indicated by the underlined segments of the J3R ORF.

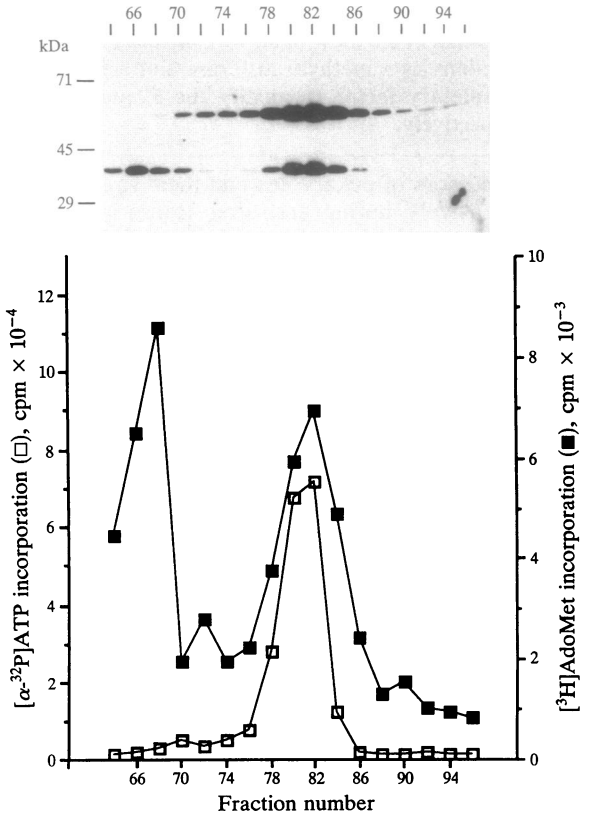

FIG. 2. Cochromatography of the mRNA (nucleoside-$O^{2'}$-)-methyltransferase with the poly(A) polymerase heterodimer and the monomeric poly(A) polymerase stimulatory factor. Soluble proteins that had been extracted from vaccinia virions and partially purified by passage through two DEAE-cellulose columns were applied to a single-stranded DNA-cellulose column and eluted with a NaCl gradient. The fractions were previously tested for poly(A) polymerase activity (□) and analyzed by SDS/PAGE and immunoblotting with antibody to VP55 and VP39 (25), and those data are reproduced here with permission. Stored fractions from the same column were analyzed for methyltransferase activity (■).

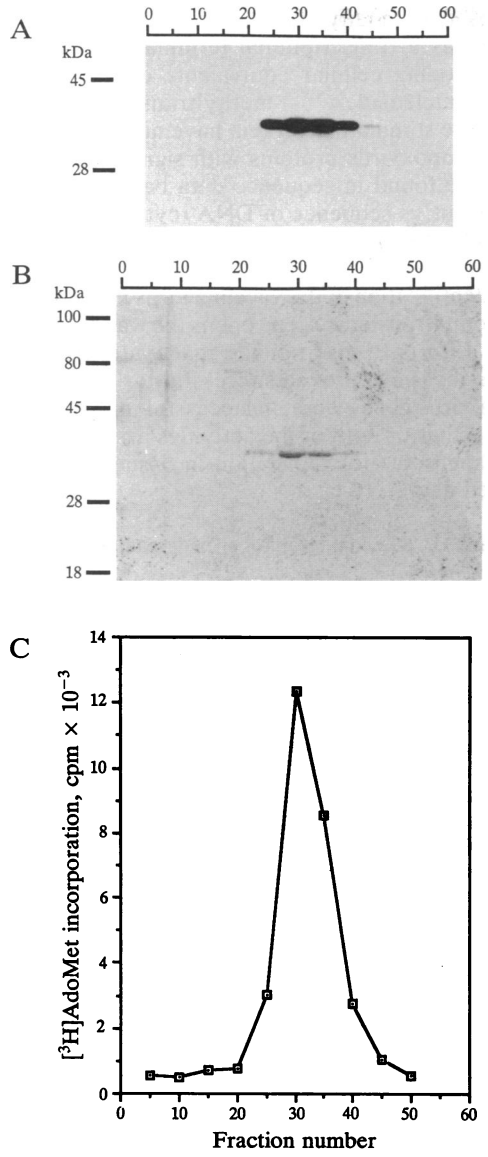

FIG. 3. Methyltransferase activity of recombinant VP39. The J3R ORF was expressed in HeLa cells by using the vaccinia virus/bacteriophage T7 system, and VP39 was purified by five chromatographic steps. Gradient fractions from a poly(A)-Sepharose column, the last step in the purification, were analyzed for VP39 by SDS/PAGE and immunoblotting with an antibody (25) to the N terminus of VP39 (*A*), for total protein by SDS/PAGE and silver staining (*B*), and for methyltransferase activity (*C*).

merase and VP55/VP39 heterodimer. Further evidence that the poly(A) polymerase holoenzyme has methyltransferase activity was obtained by centrifugation of pooled DNA-cellulose column fractions 78–84 (Fig. 2) on a glycerol gradient. The methyltransferase and poly(A) polymerase activities cosedimented as an 80- to 90-kDa heterodimeric molecule (data not shown).

Purified Recombinant VP39 Has Methyltransferase Activity. To obtain large amounts of pure VP39, the J3R ORF was overexpressed using the vaccinia virus/bacteriophage T7 RNA polymerase system (27, 28). The protein was purified to near homogeneity by five chromatographic steps, while assaying for poly(A) polymerase stimulatory activity and immunoreactivity, which coeluted (P.D.G. and B.M., unpublished work). Fractions from the last purification step, poly(A)-Sepharose chromatography, were subsequently assayed for methyltransferase activity. As shown in Fig. 3,

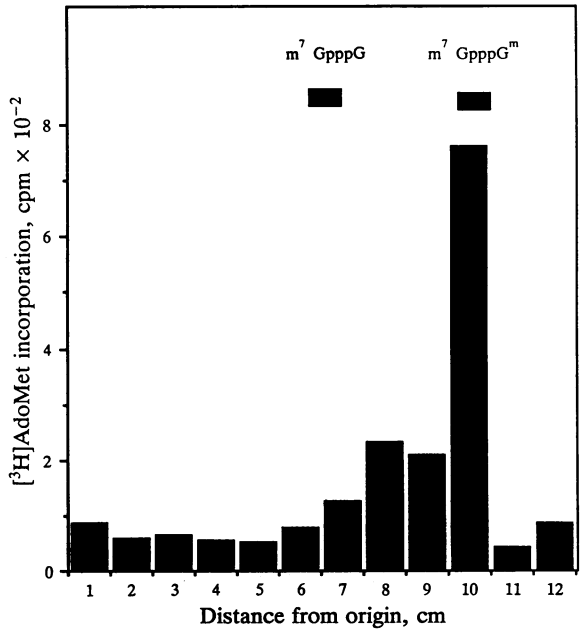

FIG. 4. Evidence for methylation of the cap structure of BMV RNA by recombinant VP39. BMV RNA, which had been methylated in the presence of Ado[*methyl*-^{3}H]Met and purified recombinant VP39, was treated with nuclease P1 and alkaline phosphatase and analyzed by chromatography on a thin-layer cellulose plate developed with isobutyric acid/0.5 M NH_4OH, 5:3. The indicated positions of marker cap dinucleotides were determined under UV light, and the plate was then cut into 1-cm strips starting from the origin. Radioactive material was determined by counting in a toluene-based scintillation fluid.

methyltransferase activity also coeluted with VP39, which comprised the major polypeptide band in a silver-stained gel.

Recombinant VP39 Specifically Methylates Cap 0 to Form Cap I Structures. The specificity of the methyl transfer catalyzed by purified recombinant VP39 was tested. The Ado[*methyl*-^{3}H]Met-labeled BMV RNA was digested with nuclease P1. The resulting mixture of 5′ nucleotides and nuclease-resistant cap structures was further digested with alkaline phosphatase to yield nucleosides and caps. The major ^{3}H-labeled product of this digestion cochromatographed with an authentic m^7GpppG^m marker on a cellulose thin-layer plate (Fig. 4). Material migrating between m^7GpppG and m^7GpppG^m probably represented the alkali-induced ring-opened form of the latter structure (5).

To characterize the methylated nucleoside, the labeled BMV RNA was digested with snake venom phosphodiesterase (which cleaves both RNA and cap structures) and alkaline phosphatase. The ^{3}H-labeled product cochromatographed with the G^m marker on a cellulose thin-layer plate (Fig. 5). The latter result is consistent with the fact that all four BMV RNAs used as substrate contain m^7GpppG caps and that methylation occurred at the 2′-*O*-position of the penultimate guanosine.

DISCUSSION

Investigations of vaccinia virus have played a major role in determining the structure (31), biosynthesis (32–35), and genetics (36, 37) of the 5′ capped end of eukaryotic mRNAs. As with other mRNAs (38), the cap is needed for efficient ribosome binding and *in vitro* translation of vaccinia virus mRNAs (39). The role of nucleoside-$O^{2'}$ methylation of the cap, however, is less clear; evidence for a translational function was obtained only by allowing mRNAs containing cap 0 and cap I structures to compete for ribosome binding (39). To permit alternative investigative approaches into the role of

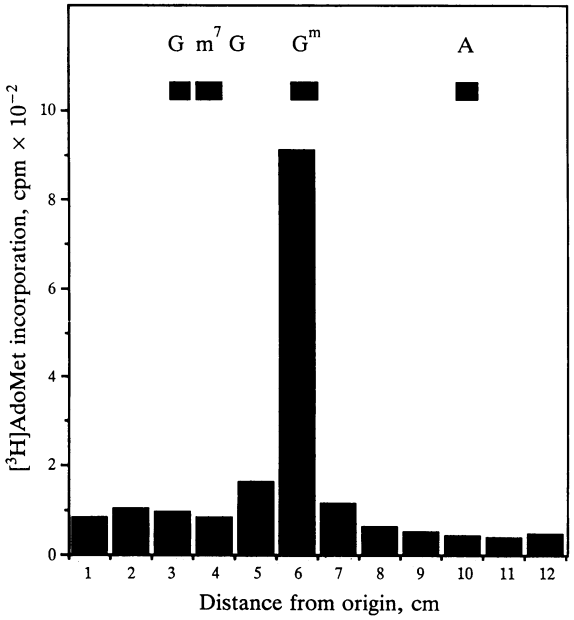

FIG. 5. Determination of the nucleoside methylated by recombinant VP39. BMV RNA, which had been methylated in the presence of Ado[*methyl*-^{3}H]Met and purified recombinant VP39, was treated with snake venom phosphodiesterase and alkaline phosphatase and analyzed by chromatography on a thin-layer cellulose plate developed with ethyl acetate/2-propanol/7.5 M NH_4OH/1-butanol, 3:2:2:1. The positions of marker nucleosides were visualized under UV light, and the plate was then cut into strips that were counted as for Fig. 4.

mRNA (nucleoside-$O^{2'}$-)-methylation, we sought to identify the vaccinia virus gene encoding the methyltransferase.

Previous studies indicated the vaccinia virus mRNA (nucleoside-$O^{2'}$-)-methyltransferase is a single subunit protein of ≈38 kDa (5). Advances in microsequencing technology (40) allowed us to obtain the sequence of several tryptic peptides derived from the purified methyltransferase. To our surprise, each of the sequences matched segments of an ORF that had recently been shown to encode the 39-kDa subunit of the vaccinia virus-encoded poly(A) polymerase (25). The vaccinia virus poly(A) polymerase consists of two subunits, VP55 and VP39, encoded by different genes. In virion extracts, VP55 exists only in association with VP39, whereas VP39 is also present as a monomer. Antibody-mediated dissociation of the poly(A) polymerase revealed that VP55 is the catalytic subunit and that VP39 greatly accelerates the formation of long poly(A) tails (25). We found that methyltransferase activity was associated with both heterodimeric and monomeric VP39. Cap-specific methyltransferase activity also was associated with recombinant VP39 that had been purified to near homogeneity from eukaryotic cells and with VP39 expressed in *Escherichia coli* (unpublished data). In contrast, no (nucleoside-$O^{2'}$-)-methyltransferase activity was associated with the VP55 monomer obtained by overexpression in eukaryotic cells (unpublished data).

The present work confirms an earlier conclusion that the mRNA (nucleoside-$O^{2'}$-)-methyltransferase is a protein of ≈38 kDa (5). The failure to appreciate that the methyltransferase also was associated with the heterodimeric poly(A) polymerase can be attributed to the entirely different methods previously used to purify the two enzymes.

The present finding that the same protein functions as a methyltransferase to modify the 5′ end of mRNA and as a poly(A) polymerase stimulatory subunit to modify the 3′ end is reminiscent of the multiple roles of the vaccinia virus capping enzyme. The latter heterodimer has triphosphatase, guanylyltransferase, and (guanine-N^7-)-methyltransferase activities that modify the 5′ end of mRNA (34) and also functions as a transcriptional termination factor (41). The genes encoding cellular equivalents of the vaccinia virus mRNA (nucleoside-$O^{2'}$-)-methyltransferase and poly(A) polymerase stimulatory protein have not yet been identified, and no nonpoxvirus proteins with significant similarities to VP39 were found in sequence data bases. The highly conserved Pro-Cys sequence of DNA (cytosine-5)-methyltransferases (42, 43) is not predicted from the sequence of VP39 ORF.

The identification of the vaccinia virus mRNA (nucleoside-$O^{2'}$-)-methyltransferase gene opens the way to further genetic studies. Efforts to insertionally inactivate the J3R ORF by methods that have proved successful for other nonessential vaccinia virus genes were unsuccessful in this case, suggesting that either or both of the activities associated with VP39 are indispensable for replication in tissue culture cells (unpublished data).

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